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14. ABSTRACT:
The association between SNCG expression and the progression of steroid dependent cancers of breast and ovary promoted us to investigate the role of SNCG in regulation of ERα. SNCG strongly stimulated the ligand-dependent transcriptional activity of ERα in breast cancer cells. A notable finding relevant to this study is that SNCG, acting as a chaperone for ER, strongly stimulated the ligand-dependent transcriptional activity of ERα, ligand-dependent cell growth, and ligand-dependent mammary tumorigenesis. Augmentation of SNCG expression stimulated the transcriptional activity of ERα and ligand-dependent growth, whereas compromising endogenous SNCG expression suppressed ERα signaling and ligand-dependent growth.

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INTRODUCTION

SNCG was first identified and cloned in PI's lab as a breast cancer specific gene, which is highly expressed in advanced infiltrating breast carcinomas but not in normal or benign breast tissue. Aberrant expression of SNCG was also associated with ovary cancer progression. Synucleins are a family of small proteins consisting of 3 known members, α synuclein (SNCA), β synuclein (SNCB), and γ synuclein (SNCG). Synucleins has been specifically implicated in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). However, studies also indicated the potential role of synucleins particularly SNCG in the pathogenesis of steroid-responsive tumors of breast and ovary. What role SNCG has in breast and ovary and how it is implicated in breast and ovary cancer remains a mystery. The association between SNCG expression and the progression of steroid dependent cancers of breast and ovary promoted us to investigate the role of SNCG in regulation of estrogen receptor ER-α.

BODY

A notable finding relevant to this study is that SNCG strongly stimulated the ligand-dependent transcriptional activity of ER-α in breast cancer cells. Since SNCG binds to ER-α, Hsp70, and Hsp90 in the absence of ligand but does not bind to ER-α and Hsp90 following ligand binding, these data suggest that SNCG is not likely to function as a coactivator involved in the post ligand binding events (such as DNA binding); but rather functions as chaperone to maintain ER-α compatible for high affinity ligand binding. We **hypotheses** that: 1) one of the critical functions of SNCG on breast cancer pathogenesis is to stimulate ER-α transcriptional activity; and 2) SNCG stimulates ER-α activation by participating in Hsp-based multiprotein chaperone system for efficient activation of steroid receptors; and this stimulation of ER-α activation is mediated at the stage of hormone binding.

SA1. To determine the mechanism of SNCG-stimulated ER-α activation; particularly we will focus on Hsp-based multiprotein chaperone complex for ER-α and to determine whether the SNCG-stimulated hormone dependent ER-α transactivation is mediated at the stage of hormone binding (Finished. Cancer Res 63: 3899-3903, 2003; Cancer Res 64: 4539-4546, 2004).

SA1-1. SNCG strongly stimulated the ligand-dependent transcriptional activity of ERα in breast cancer cells (Cancer Res 63: 3899-3903, 2003). Augmentation of SNCG expression stimulated transcriptional activity of ERα, whereas compromising SNCG expression suppressed ERα signaling. The SNCG-stimulated EFα signaling was demonstrated in three different cell systems:

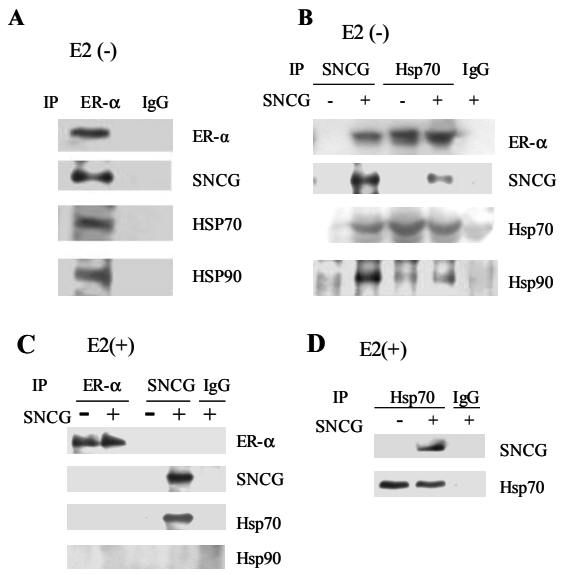
1. *Overexpression of SNCG stimulated transcriptional activity of ERα.* Transfection of SNCG gene into the SNCG-negative and ER-positive MCF-7 cells did not affect ERα expression but significantly stimulated E2-mediated activation of ERα. Overexpression of SNCG gene in MCF-7 cells increased E2-stimulated reporter activity 3.2-fold over the SNCG-negative control cells. The SNCG-stimulated transcriptional activity of ERα was ligand-dependent, because SNCG had no significant effect on the transcriptional activity of ERα in the absence of E2.
2. *Co-transfection of SNCG and ERα into SNCG-negative and ERα-negative MDA-MB-435 cells*. Treatment of ER-α-transfected MDA-MB-435 cells with E2 activated reporter activity, indicating the functional transcriptional activity of the transfected ERα gene. A significant stimulation of ERα signaling by SNCG was observed in MDA-MB-435 cells when the cells

were co-transfected with ERα and SNCG constructs. SNCG increased ligand-dependent transcriptional activity 3.7-fold over the control cells.

3. *Antisense blocking SNCG expression in ERα-positive and SNCG-positive T47D cells*. The effect of SNCG expression on ERα transactivation was further demonstrated by inhibiting endogenous SNCG expression with SNCG antisense mRNA in T47D cells that express high levels of SNCG. Stable transfection of the SNCG antisense construct into T47D cells significantly reduced SNCG expression to 25% of that in control T47D cells. While E2 significantly stimulated the reporter activity in the control T47D cells, inhibition of SNCG expression reduced E2-responsive activity in two stable antisense-transfected T47D cell lines, AS-1 and AS-3 cells, to 21% and 13% of that in control T47D cells, respectively. Treatment of T47D cells with E2 resulted in a 25-fold increase over the non-treated cells. However, only 3.3- and 5.2-fold increase was observed in the AS-3 and AS-1 cells, respectively.

SA1-2. Mechanisms by which SNCG stimulates ER signaling and ligand-dependent growth (Cancer Res 64: 4539-4546, 2004). It is well documented that the activation of steroid receptors is modulated by a heterocomplex with several molecular chaperones, particularly with Hsp90 and Hsp70, which associate with the unliganded steroid receptors and maintain them in a high affinity hormone-binding conformation. The chaperone-like activity of synucleins has been demonstrated in the cell-free system. Because SNCG stimulated ligand-dependent transcriptional activity of ERα, which can be blocked by antiestrogen, we reason that SNCG may have a chaperone activity and participate in Hsp-based multiprotein chaperone complex for ERα.

SNCG is a novel Hsp70-associated chaperone, participated in the Hsp-based multiprotein chaperone complex for ERα. SNCG bound to the unliganded form of ER-α, Hsp90, and Hsp70. We investigated if SNCG can physically and functionally interact with ERα, Hsp70, and Hsp90 in SNCG transfected MCF-7 cells by co-immunoprecipitation assays. IP of ERα co-precipitated SNCG, Hsp70 and Hsp90 in the absence of estrogen (***Fig. 1A***) and *vice versa* (***Fig. 1B***), indicating that SNCG participated in a heterocomplex with Hsp90, Hsp70, and ER-α in the absence of estrogen. However, SNCG dissociated from ERα after the cells were treated with E2 (***Fig. 1C***). The binding pattern of SNCG to the unliganded ERα is same to that of Hsp90 and Hsp70, which only binds to the unliganded ER-α (34). Similar to its binding pattern to ERα, SNCG only bound to Hsp90 in the absence of estrogen (***Fig. 1B***). After cells were treated with E2, the liganded ER-α dissociated from SNCG, Hsp70, and Hsp90 (***Fig. 1C***). However, in contrast to its binding pattern to ERα and Hsp90, SNCG was found to bind to Hsp70 under the conditions both without (***Fig. 1B***) and with E2 (***Fig. 1D***), indicating that SNCG binds to Hsp70 constitutively regardless of whether Hsp70 is associated with ERα.



SNCG increases ligand binding by ERα. It has been proposed that Hsp-based chaperone complex inactivates the ER's transcriptional regulatory capabilities and maintains the ER in a conformation competent for steroid binding. We examined whether the SNCG-stimulated hormone dependent ERα transactivation results from increased estradiol binding by the receptor.

As demonstrated in **Fig. 2**, SNCG significantly enhanced ligand-binding by ER. The biggest increases in the ligand binding were observed at the lower concentration range of 0.0025-1 nM of ligand. At the concentration of 1 nM of E2, the ligand binding was increased 160% in the SNCG-positive cells vs. the SNCG-negative control cells. Scatchard analysis revealed two binding sites (**Fig. 3**). The high affinity state was apparent in a linear plot between 0.0025nM and 1 nM of estradiol, and the low affinity state between 1 nM and 10 nM of estradiol. SNCG overexpression significantly enhanced the high affinity state of ER in MCF-7 cells, resulting in a 2.3-fold increase in high affinity binding capacity. While the high affinity binding capacity in MCF-7-neo cells was saturated when the bound E2 reached at 0.06 pmol/mg protein, the high affinity binding capacity in MCF-7-SNCG cells was saturated when the bound E2 reached at 0.14 pmol/mg protein. These data indicate that SNCG affects ER-α signal transduction pathway at the step of ligand binding by increasing the number of high affinity ligand-binding sites.

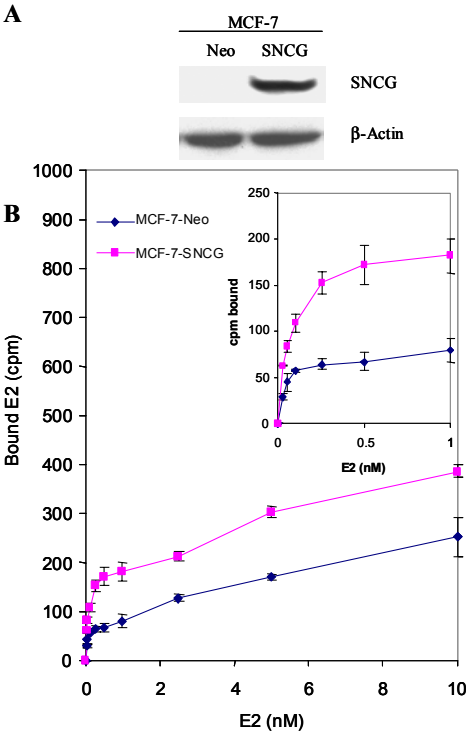


Fig. 2. Estrogen-binding capability for ER in SNCG-transfected MCF-7 cells (MCF-7-SNCG) and control neo-transfected cells (MCF-7-Neo). Cells were transiently transfected with pCI-SNCG or pCI-neo plasmids. The transfected cells were enriched with Neomycin selection for 12 days before the hormone binding assay. **A**, Western blot analysis of SNCG expression in pooled SNCG-transfected MCF-7 cells after selection with G418. **B**. Titration of ^{3}H-E2 in MCF-7-Neo and MCF-7-SNCG cells. *Inset*, enlarged view of ^{3}H-E2 titration from 0-1 nM of ligand.

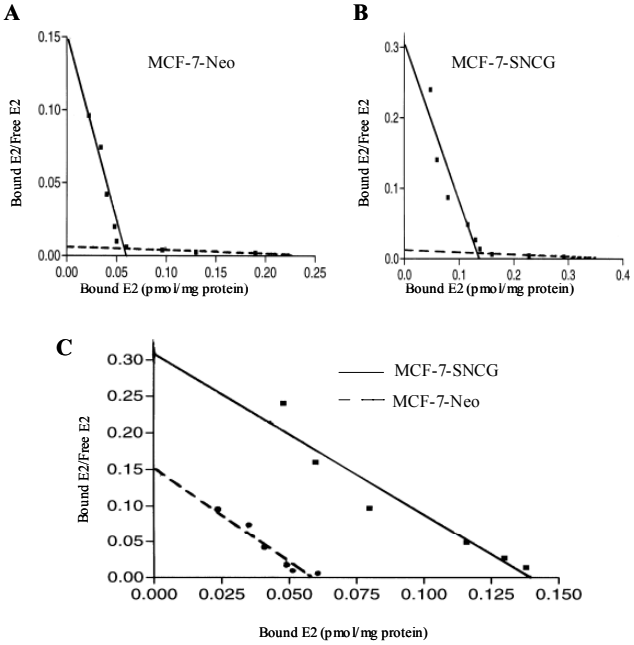


Fig. 3. Scatchard analysis of the ligand-binding by ER from MCF-7-Neo cells (**A**) and MCF-7-SNCG cells (**B**). *Unbroken line*, high affinity sites; *broken line*, low affinity sites. **C,** The high affinity sites of MCF-7-Neo and MCF-7-SNCG cells. Specific binding was determined by subtracting the non-specific binding from samples incubated with 100-fold excess of non-labeled E2. Each data point is the mean ± SD of triplicate samples.

SA2. To study the biological relevance of SNCG-stimulated ER-α signaling to hormone-dependent tumorigenesis. The major goal of this specific aim is to determine if SNCG expression

in breast cancer cells stimulate estrogen-mediated tumor growth. We will determine if expression of SNCG in breast cancer cells will stimulate estrogen-mediated tumor growth in nude mice. This will be investigated in MCF-7 and T47D cells.

SA2-1. Stimulation of estrogen-mediated cell growth. The SNCG-stimulated ERα transcriptional activity is consistent with its stimulation of the ligand-dependent cell growth. It has been demonstrated in different systems that the interaction between SNCG and ERα stimulated cell growth.

MCF-7 cells. The cellular proliferation of two stably SNCG-transfected MCF-7 cell clones were compared with that of SNCG-negative neo clones. ***Fig 4A*** shows that while SNCG had no significant effect on the proliferation of SNCG-transfected cells compared to MCF-neo cells in the absence of E2, overexpression of SNCG significantly stimulated the ligand-dependent proliferation. Treatment of neo clones with E2 stimulated an average cell proliferation 2.4-fold over controls. However, E2 treatment of SNCG clones resulted in an average of 5.4-fold increase in the proliferation vs. controls, suggesting that SNCG expression renders the cells more responsive to E2-stimulated cell proliferation.

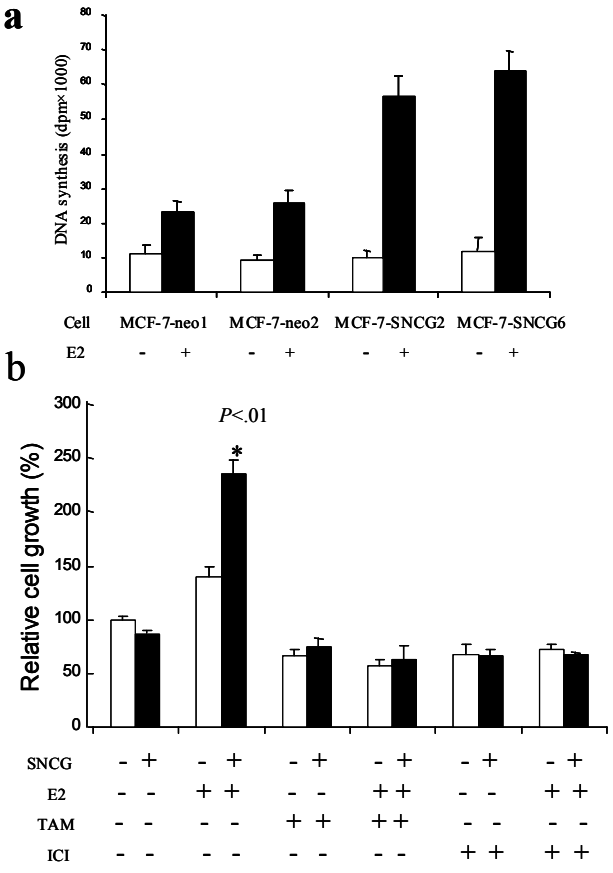


Fig. 4. SNCG stimulated ligand-dependent cell proliferation. For all experiments, cells were cultured and synchronized in the ligand-free Conditioned Cell Culture medium for 4 days before the hormone treatments. **A,** Stimulation of cell proliferation by SNCG overexpression. Cells were treated with or without 1 nM E2 for 24 hours. Cell proliferation was measured by ^{3}H- thymidine incorporation. Data are means ± SD of three cultures. **B**, Effect of antiestrogens on SNCG-stimulated cell growth. Cells were treated with or without 1 nM of E2, 1 μM of tamoxifen, or 1 μM of ICI for 6 days before harvesting. Media were changed every two days with fresh estrogen and antiestrogens. Cell growth was measured using a cell proliferation kit (XTT). Data are the mean ± SD of quadruplicate cultures. Open bar represents MCF-neo1 cells; closed bar represents MCF-SNCG6 cells.

Blocking endogenous SNCG in T47D cells by antisense construct. Soft agar colony assays demonstrated that the anchorage-independent growth of T47D cells expressing SNCG antisense mRNA was significantly suppressed. When cells were cultured in soft agar without E2, there were very few colonies formed in both T47D group and T47D-SNCG antisense group. Treatment of T47D cells with E2 resulted in a 19-fold increase of colonies over the non-treated cells. However, treatment of T47D cells expressing SNCG antisense mRNA with E2 resulted in only 3-fold increase over the non-treated cells (***Fig. 5***). These data demonstrated that inhibition of endogenous SNCG expression dramatically diminished the cell growth in response to estrogen.

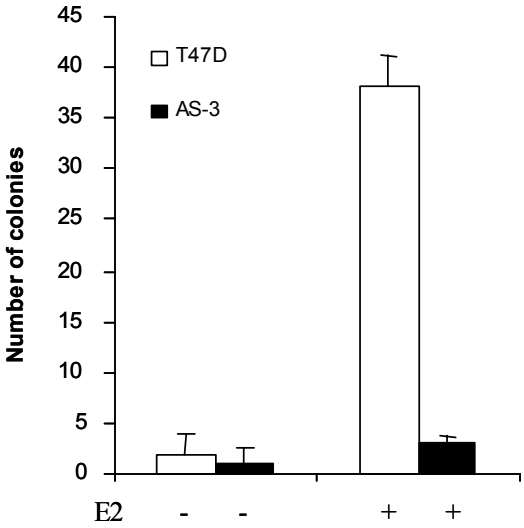


Fig. 5. Effect of inhibiting endogenous SNCG expression on soft agar colonies formation capability of T47D cells. T47D and SNCG antisense stably transfected AS-3 clone were cultured into the top layer soft agar and treated with or without 1 nM of E2 as described in Materials and Methods. The number of colonies was counted after 2 weeks of plating using a Nikon microscope at 100× amplification. Triplicate wells were assayed for each condition.

SA2-2. Stimulation of estrogen-dependent mammary tumorigenesis by SNCG (Cancer Res 64: 4539-4546, 2004). An orthotopic nude mouse model was used to study the effects of SNCG on tumor growth. Two independent experiments under different conditions were done to determine the effects of SNCG on mammary tumorigenesis.

We first analyzed the tumorigenesis in response to E2 in the non-ovariectomized intact mice. It was previously demonstrated that the circulating E2 level in the non-ovariectomized mice is 26 pg/ml (31), which compares to the low levels found in postmenopausal women (32). To override the endogenous levels of estrogen, all the mice were supplemented with E2 (0.72 mg/pellet) one day before injection of 5 x10^6 cells. After a lag phase of 8-10 days, 29 of 32 (91%) injections in the mice given implants of SNCG positive MCF-SNCG2 and MCF-SNCG6 cells developed tumors. In contrast, only 21 of 32 (66%) injections in the mice given implants of SNCG negative MCF-neo1 and MCF-neo2 cells developed tumors (***Table 1***).

Table 1. Effects of SNCG expression on tumor incidence and tumor growth of MCF-7 cells

		E2	*Tumor incidence*	*Tumor volume (mm^3)*	
Experiment	Group		tumor/total (%)	Day 21	Day 35
	MCF-SNCG2	+	14/16 (88)	234 ± 69	1850 ± 280
	MCF-SNCG6	+	15/16 (94)	351 ± 78	2521 ± 390
	MCF-neo1	+	10/16 (63)	101 ± 39	491 ± 92

Cells were injected into the mammary fat pads, and tumor volumes and tumor incidence were determined as described in Materials and Methods. Each mouse received two injections. Tumor volumes were measured at 21 days and 35 days following cell injection and are expressed as means ± SEs (number of tumors assayed). All the non-ovariectomized mice received an estrogen implantation one day before the cell injection. There were total 16 injections for 8 mice in each group, and each injection had 5 x 10^6 cells. Statistical comparisons for pooled SNCG positive clones relative to pooled SNCG negative clones indicated $P < 0.01$ for the mean tumor sizes and $P < 0.05$ for the tumor incidence. Statistical comparison for primary tumors was

analyzed by Student's *t* test. A chi-square test was used for statistical analysis of tumor incidence.

The tumor growths in MCF-SNCG clones were significantly stimulated. At 35 days following tumor cell injection, the size of MCF-SNCG6 tumors, which expressed relative high level of SNCG mRNA, was 4.8-fold of that in parental MCF-neo2 tumors and 3.7-fold of that in MCF-neo1 tumors. In addition, the tumor incidence was also increased. With 16 injections, while 15 implants in MCF-SNCG6 cells developed tumors, only 10 implants from MCF-neo1 and 11 implants from MCF-neo2 developed tumors, respectively. The tumor growth of MCF-SNCG2 cells was also significantly stimulated, with 3.5-fold and 2.7-fold increase in tumor size as compared to MCF-neo2 and MCF-neo1 tumors, respectively. ***Fig. 6*** shows growth kinetics. After a slow growth phase of 14 days, tumor growth of MCF-SNCG2 and MCF-SNCG6 clones were significantly enhanced as compared to that of MCF-neo1 and MCF-neo2 clones. Thus, the tumorigenesis of MCF-7 cells in response to E2 was significantly stimulated by the SNCG overexpression.

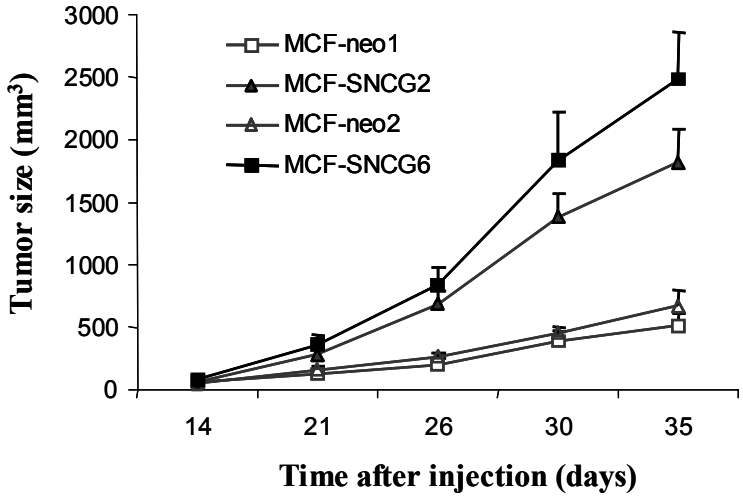


Fig. 6. Stimulation of MCF-7 tumor growth by SNCG. Each of the eight E2-supplemented non-ovariectomized mice in each group received two injections, one on each side, in the mammary fat pads between the first and second nipples. Tumor size was determined by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each clone at each time point. Each point represents the mean of tumors ± SE (bars).

We then analyzed the requirement of E2 for SNCG-mediated tumor stimulation under more stringent conditions including use of ovariectomized mice and reduction of injected tumor cells from 5 x 10^6 to 1.5 x 10^6. As shown in ***Table 2***, when the number of injected MCF-neo1cells was reduced, the tumor incidence was greatly reduced from 63% to 0% both in the absence and presence of E2 supplement for up to 4 weeks, indicating a non-tumorigenic condition for SNCG-negative MCF-7 cells even with estrogen stimulation for 4 weeks. At the week 7, a 30% of tumor incidence with small tumor size (44 mm^3) was observed. However, for MCF-SNCG6 cells, although a same non-tumorigenic phenotype was observed under the conditions with reduced cell number and in the absence of E2, when E2 was supplemented, the tumor incidence reached to 90% at week 3 after cell inoculation, which is similar to 94% of tumor incidence in the experiment with higher injected cell numbers (***Table 1***). Furthermore, the tumor size of SNCG-positive cells is 4.4 fold over that of SNCG-negative cells. These data indicate that estrogen is necessary for SNCG-mediated tumor stimulation in the xenograft model.

Table 2. Stimulation of estrogen-mediated tumorigenesis by SNCG

Group	E2	Tumor Incidence (%)							Tumor Vol (mm^3)
	Week	1	2	3	4	5	6	7	Week 7
MCF-neo1	-	0	0	0	0	0	0	0	
MCF-neo1	+	0	0	0	0	20	30	30	44±10
MCF-SNCG6	-	0	0	0	0	0	0	0	
MCF-SNCG6	+	40	80	90	90	90	90	90	194±35

Ovariectomized mice were treated with or without E2 pellet. There were total 10 injections for 5 mice in each group, and each injection had 1.5 x 10^6 cells. Only measurable tumors were used to calculate the mean tumor volume. Statistical comparisons for SNCG positive clone relative to SNCG negative clone indicated $P < 0.001$ for both tumor incidence and mean tumor sizes in the presence of E2.

KEY RESEARCH ACCOMPLISHMENTS AND REPORTABLE OUTCOMES

1. SNCG strongly stimulated the ligand-dependent transcriptional activity of ERα in breast cancer cells. Augmentation of SNCG expression stimulated transcriptional activity of ERα, whereas compromising SNCG expression suppressed ERα signaling.
2. Stimulation of estrogen-mediated cell growth. The SNCG-stimulated ERα transcriptional activity is consistent with its stimulation of the ligand-dependent cell growth.
3. SNCG-mediated stimulation of ERα signaling and cell growth can be inhibited by antiestrogens.
4. Stimulation of estrogen-dependent mammary tumorigenesis by SNCG.
5. SNCG is a novel Hsp70-associated chaperone**,** participated in the Hsp-based multiprotein chaperone complex for ERα, and increased ligand binding by ERα.

CONCLUSIONS

Although synucleins are emerging as central players in the formation of pathologically insoluble deposits characteristic of neurodegenerative diseases, γ Synuclein (SNCG), previously identified as a breast cancer specific gene (BCSG1), is also highly associated with breast or ovarian cancer progression. However, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. Here we demonstrated a chaperone activity of SNCG in the heat shock protein-based multiprotein chaperone complex for stimulation of ER-α signaling. As an ER-α-associated chaperone, SNCG participated in Hsp-ER-α complex, enhanced the high affinity ligand-binding capacity of ER-α, and stimulated ligand-dependent activation of ER-α. The SNCG-mediated stimulation of ER-α transcriptional activity is consistent with its stimulation of mammary tumorigenesis in response to estrogen. These data indicate that SNCG is a new chaperone protein in the Hsp-based multiprotein chaperone complex for stimulation of ligand-dependent ER-α signaling and, thus, stimulates hormone responsive mammary tumorigenesis.